# Cloning and expression profiling of heat shock protein DaHSP23 gene in the winter and summer diapause pupae of the onion maggot, *Delia antiqua* (Diptera: Anthomyiidae)

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**Abstract**: 【Aim】 The sHSPs are a diverse of molecular chaperones with molecular weights ranging from 12 to 43 kDa in different species. These proteins have been shown to be crucial components of the diapause of some species, but need to be further elucidated. The study aims to clone and characterize the *sHSP* gene of *Delia antiqua*, of which orthologs were earlier reported to be diapause-related, and to investigate the expression patterns of the gene during both winter and summer diapauses of the species. 【Methods】 A sHSP gene was cloned from *D. antiqua* using RACE-PCR, and characterized through homology, domain and phylogenetic analyses. The existence of the intron was investigated with genome sequencing around the gene. The expression level of this gene during both winter diapause (WD) and summer diapause (SD) was detected real-time qPCR, and the expression difference was compared to reveal the association with diapause development. 【Results】 The cDNA of *sHSP* cloned from *D. antiqua*, which is named *DaHSP*23 (GenBank accession no. HQ392521.1), is 904 bp in length, encoding 186 amino acids with a molecular mass of 20.9 kDa and a theoretical isoelectric point of 6.42. *DaHSP*23 shares more than 66% amino acid identity with the known HSPs of other dipteran insects and is homologous with the known HSP23 genes of other dipteran insects. Genome sequencing around the gene revealed that it is intronless. The expression of *DaHSP*23 was up-regulated in both SD and WD pupae, with higher expression level detected in the late maintenance stage, and down-regulated when those diapauses were terminated. 【Conclusion】 The patterns of *HSP*23 regulation are substantially different between different developmental stages of both SD and WD, and its regulation in diapause might be species-specific. *DaHSP*23 might play an essential role in these two types of diapauses in *D. antiqua*.

**Key words**: *Delia antiqua*; diapause; heat shock protein (HSP); gene cloning; expression pattern; real-time qPCR

## 1 INTRODUCTION

Heat shock proteins (HSPs) are conserved proteins involved in multiple cellular processes, including protein folding, targeting, and translocation across membranes (Neupert, 1997; Hartl and Hayer-Hartl, 2002). According to the homology and molecular weights, HSPs can be mainly classified into three main families: HSP90 (85 – 90 kDa), HSP70 (68 – 73 kDa) and the small HSP (sHSP) (12 – 43 kDa) (Denlinger *et al.*, 2002).

sHSPs contain an α-crystalline domain with molecular weights of 12 – 43 kDa, depending on the variable N- and C-terminal extensions (MacRae, 2000; Taylor and Benjamin, 2005). Studies in different experimental systems have revealed a variety of functions for the sHSP under stress conditions, including basic chaperoning activity (Ehrnsperger *et al.*, 1997; Haslbeck *et al.*, 1999), cytoskeleton protection (Lavoie *et al.*, 1993) and modulation of the apoptotic process (Bruey *et al.*, 2000). Despite the classical definition of heat shock proteins as polypeptides induced by stress, cell-specific expression of sHSPs in the absence of stress has been reported during the development of a wide range of organisms such as *Caenorhabditis elegans* (Ding *et al.*, 2000), *Drosophila melanogaster* (Glaser *et al.*, 1986; Marin *et al.*,1993; Michaud *et al.*,1997), *Xenopus laevis* (Lang *et al.*, 1999), *Mus musculus* (Gernold *et al.*, 1993; Tanguay *et al.*,

基金项目: "两江学者"计划专项经费(20136666); 国家自然科学基金项目(31372265); 国际原子能机构 IAEA 研究项目(18268/R2); 科技基础性工作专项重点项目(2015FY210300); 重庆市教委科学技术研究项目(KJ1400517); 重庆师范大学青年基金项目(2011XLS33)

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收稿日期 Received: 2015-11-30; 接受日期 Accepted: 2016-03-02

1993; Armstrong *et al.*, 2001), and human being (Jantschitsch *et al.*, 1998).

Diapause was developed by insects and arthropods to survive from unfavorable seasons. *Delia antiqua* can be induced into both summer diapause (SD) and winter diapause (WD) after head evagination in pupal stage, and easy to be reared in laboratory; therefore, it is regarded as a good model for diapause study (Ishikawa *et al.*, 1987, 2000). The diapause program was suggested to associate with the expression of diapause-specific genes (Denlinger, 2002). The heat shock proteins, including sHSP, HSP60, HSP70 and HSP90 (Macrae, 2010), are of great importance in understanding the molecular mechanisms of diapause.

In response to temperature and stresses, *HSP*90 and *HSP*70 were expressed in the summer and winter diapause pupae of *D. antiqua* (Chen *et al.*, 2005a, 2005b, 2006). In the pupal diapause of the flesh fly *Sarcophaga crassipalpis*, 23 kDa heat shock protein (HSP23) gene transcripts are expressed at very high levels as part of the normal diapause program, indicating that HSP23 plays an essential role during this over-wintering developmental arrest (Yocum *et al.*, 1998). However, in the blow fly *Lucilia sericata*, the level of *HSP*23 transcripts was found to be consistently low, and no difference in its expression level was observed between normal condition and diapause, indicating that expression of *HSP*23 is not regulated in response to diapause (Tachibana *et al.*, 2005). This inconsistency needs to be elucidated with additional species and different types of diapause. To our knowledge, no study has as yet been carried out to explore the expression of *HSP*23 during insect summer diapause. It will be interesting to compare the expression patterns between SD and WD.

In the present study, we cloned the full-length cDNA of *HSP*23 gene (*DaHSP*23) from the onion maggot, *D. antiqua*, compared the expression patterns of *DaHSP*23 among summer-, winter- and non-diapausing pupae using real-time quantitative RT-PCR, and discussed the *HSP*23 regulation in relation to diapause.

# 2 MATERIALS AND METHODS

## 2.1 Insect rearing and diapause induction

The non-diapausing (ND) *D. antiqua* was reared on an artificial diet at 20℃ with a 16L∶8D photoperiod and 50% – 70% relative humidity as described by Chen *et al.* (2010). To induce SD, larvae were maintained at 25℃ with the 16L∶8D photoperiod. Newly formed pupae were kept under the same conditions as larvae until 10 days after pupation (D10) and then transferred to 16℃ and 16L∶8D photoperiod to trigger diapause termination (Chen *et al.*, 2010). To induce WD, larvae were reared at 15℃ with the 12L∶12D photoperiod (Ishikawa *et al.*, 2000), and pupae were kept under this condition throughout. Pupae collected at different developmental stages of ND, SD and WD were frozen in liquid nitrogen and stored at −80℃ for RNA extraction.

## 2.2 RNA extraction, cDNA synthesis, and PCR amplification

Total RNA was isolated with the RNeasy Mini Kit (QIAGEN) according to the supplier's instructions. The first-strand cDNA was synthesized from total RNA using oligo (dT) primer (TaKaRa) and Superscript™ reverse transcriptase (Invitrogen) according to the supplier's instructions. A 284 bp cDNA fragment of *HSP*23 was amplified by PCR from cDNA using degenerate primers (Table 1), which were designed based on consensus sequences (number from 202 to 485, Fig. 1.) from several dipterans. PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 35 cycles at 94℃ for 30 s, 55℃ for 30 s and 72℃ for 60 s; and 1 cycle at 72℃ for 10 min. The expected DNA band was excised from the agarose gel, purified using a DNA gel extraction kit (Bioteke, Beijing) and cloned into a pMD-19T Vector (TaKaRa). Plasmids were isolated from positive clones and sequenced by Shanghai Songon Company.

## 2.3 Amplification of the full-length *HSP*23 cDNA

To obtain the full-length *HSP*23 cDNA, we performed 5′ and 3′ rapid amplification of cDNA ends (5′ RACE and 3′ RACE, respectively) using SMART™ RACE kit (Clontech) according to the manufacturer's instructions. For 5′ RACE, gene-specific primers Gene Races 5′-R and Gene Races 5n′-R were designed based on the 284 bp cDNA fragment. The amplification was performed with 1 μL of 5′-ready-cDNA using Universal Primer Mix (UPM, Clontech) and Gene Races 5′-R, and then the nested PCR was carried out with Gene Races 5n′-R and Nested Universal Primer A (NUP, Clontech). The PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 35 cycles at 94℃ for 30 s, at 60℃ for 30 s, and at 72℃ for 60 s; and 1 cycle at 72℃ for 10 min. For the 3′ RACE, the cDNA was synthesized according to the manufacturer's protocol [TaKaRa RNA PCR Kit (AMV) Ver. 3.0]. Gene-specific primers Gene Races 3′-F and Gene Races 3n′-F were designed also based on the 284 bp cDNA fragment. The amplification was performed with 1 μL of 3′-ready-cDNA using M13 Primer M4 (M4,

**Table 1 Primers used in this study**

| Primer name | Primer sequences (5′ – 3′) | Use of primers |
|---|---|---|
| Hsp23F | TTYCARGTNTGYATGGAYGT | Amplification of *DaHsp*23 fragment |
| Hsp23R | GGNCSNACYTGYTGDAYTG | |
| Gene Races 3′-F | CGAGGGTAAACATGAGGAACGGGAA | Amplification of *DaHsp*23 3′ end |
| Gene Races 3n′-F | ACCCGACACTTTGTACGTCGCTATGC | |
| Gene Races 5′-R | GCATAGCGACGTACAAAGTGTCGGG | Amplification of *DaHsp*23 5′ end |
| Gene Races 5n′-R | TTCCCGTTCCTCATGTTTACCCTCG | |
| Hsp23-CDSF | ATGGCAAACTTACCACTTCTTTTGA | Amplification of *DaHsp*23 genomic DNA sequence |
| Hsp23-CDSR | TTACAATTTCGATGGAGGCTGTTCC | |
| Hsp23QF | ACACTTTGTACGTCGCTATGCTT | *DaHsp*23 real-time qPCR |
| Hsp23QR | ACGTTCGTTGCTGGATTTATCT | |

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-132                                                GAA GAA AAC AAA ACC AAA GCA AAT CAA ATC
-102 AAC AAA GTC AAA TTA ATA TTT TTA TTT AAA AAC TTT AAA TAA TTG TTA AAA
 -51 AGT GTT GAA ATT TTA AAT ATT ATT AAA TAA TAA AAC AGT AAA TCT ATA AAA
   1 ATG GCA AAC TTA CCA CTT CTT TTG AGT TTG GCT AAC GAT CTC AAT CGC CTT
   1  M   A   N   L   P   L   L   L   S   L   A   N   D   L   N   R   L
  52 TCC TTG GCT GCT TCG CCG TTT TAT GAA TCA CCA ATT CAC TAT TTG CGC CAT
  18  S   L   A   A   S   P   F   Y   E   S   P   I   H   Y   L   R   H
 103 CCT GGC TAT TTG GCC TTG CTG AAC GCT GAT CAA CCA CAA CAA CTA CGA AAA
  35  P   G   Y   L   A   L   L   N   A   D   Q   P   Q   Q   L   R   K
 154 CAT GAT AAG GAA TCG TCT GGT CCA CTA GCC ACC GTT GGC AAG GAT GGT TTC
  52  H   D   K   E   S   S   G   P   L   A   T   V   G   K   D   G   F
 205 CAG GTA TGC ATG GAT GTT CAA CAA TTC AAG CCC AGT GAG TTG AAT GTC AAA
  69  Q   V   C   M   D   V   Q   Q   F   K   P   S   E   L   N   V   K
 256 GTC ATC GAC AAT TGT GTA ATT GTC GAG GGT AAA CAT GAG GAA CGG GAA GAT
  86  V   I   D   N   C   V   I   V   E   G   K   H   E   E   R   E   D
 307 GAT CAT GGC TTT ATT ACC CGA CAC TTT GTA CGT CGC TAT GCT TTA CCC AAA
 103  D   H   G   F   I   T   R   H   F   V   R   R   Y   A   L   P   K
 358 GGT TAT GAT TCC AAT AAA GTT CAA TCC ACT TTA TCC TCG GAT GGT GTT TTA
 120  G   Y   D   S   N   K   V   Q   S   T   L   S   S   D   G   V   L
 409 ACA GTT AGC GTA CCC AAA CCT CAA ATC GAA GAT AAA TCC AGC AAC GAA CGT
 137  T   V   S   V   P   K   P   Q   I   E   D   K   S   S   N   E   R
 460 CAA ATT CAA ATT CAA CAA GTT GGT CCA GCA CAT TTG AAT GTC AAA GAA AAT
 154  Q   I   Q   I   Q   Q   V   G   P   A   H   L   N   V   K   E   N
 511 CCT AAA GAA GAA AAG GAA AGT GAT AAG GAA CAG CCT CCA TCG AAA TTG TAA
 171  P   K   E   E   K   E   S   D   K   E   Q   P   P   S   K   L   *
 562 AAG TCT AAC ACT TAT TGG CAT TTG TTT GAT TAT TTG AGA GTA AAT AGT CTG
 613 TTT AAT TTT AAG TAA AAA TTT TAA TTA TAC TTG TTA ACC AAA ATT TTA TAC
 664 GAC TAT TTA TAA AAA TTA AAA AGA ACT GAA TTT TGT TTT AAA AAT AAA TAA
 715 AAT TTA TAA GTA CCA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
 766 AAA AAA  A
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Fig. 1 cDNA and deduced amino acid sequences of *HSP*23 from *Delia antiqua* (*DaHSP*23)

The start codon ATG and stop codon TAA are indicated in bold. A putative polyadenylation site (AATAAA) is underlined. The numbers on the left indicate the positions of nucleotides and amino acids in the sequences.

TaKaRa) and HSPF2, and then the nested PCR was carried out with HSPf22 and M13 Primer M4 (M4, TaKaRa). The PCR conditions were as follows: 1 cycle at 94℃ for 3 min; 35 cycles at 94℃ for 30 s, at 59℃ for 30 s, and at 72℃ for 30 s; and 1 cycle at 72℃ for 10 min.

The obtained 5′- and 3′-RACE PCR products were cloned, sequenced and assembled into the full-length *HSP*23 cDNA sequence using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

## 2.4 Cloning of *HSP*23 gene from *D. antiqua* genomic DNA

Genomic DNA was isolated from non-diapausing pupae of *D. antiqua* using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Japan) according to the manufacturer's instructions. The concentration and quality of DNA were verified by spectrophotometer and electrophoresis on 1.0%

agarose gel. In order to determine whether the *DaHSP*23 gene contains introns in the coding region, the genomic DNA sequence for *DaHSP*23 was amplified from genomic DNA (100 ng). The amplification was performed using a pair of specific primers Hsp23-CDSF and Hsp23-CDSR, and the amplification conditions were as follows: 1 cycle at 94℃ for 3 min; 35 cycles at 94℃ for 30 s, at 59℃ for 45 s, at 72℃ for 1 min; and 1 cycle at 72℃ for 10 min. The genomic DNA sequence was then cloned and sequenced for *DaHSP*23 intron analysis.

### 2.5 Sequence and phylogenetic analysis

The nucleotide and protein sequences were edited, and the theoretical molecular weight and isoelectric point of sHSPs were predicted using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The deduced amino acid sequence of the *DaHSP*23 cDNA was used as query to perform a global search in GenBank non-redundant protein database by BlastP program at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST), and then the dipteran homologues of sHSPs were selected and analyzed. Multi-sequence alignment was generated using Clustal X and DNAStar 5.0 software. The functional motif and sites were deduced in reference to homologous sHSP of *D. melanogaster*. The phylogenetic analysis using neighbor-joining method with *p*-distance model was performed with MEGA 4.0. The homologous sHSP of *Caenorhabditis elegans* (NP_001023957.1) was used as the outgroup, and a total of 1 000 bootstrap replications were used to test the topology.

### 2.6 Real-time qPCR

Total RNA was isolated from 30 mg of pupae using a RNeasy Midi/Maxi kit (Qiagen) and treated with RNase-Free DNase I (Qiagen). cDNA was subjected to reverse transcription from total RNA using random primers with a RNA PCR kit (TaKaRa) and purified with a SUPREC™-02 kit (TaKaRa). The *HSP*23 fragment (133 bp) was amplified using gene-specific primers Hsp23QF and Hsp23QR. The 18S rRNA gene was used as a reference. Primers for 18S rRNA were the same as those used by Chen *et al.* (2005a), which amplifies a 333 bp fragment. One cDNA sample, diluted to 1, $5^{-1}$, $5^{-2}$, $5^{-3}$, $5^{-4}$ and $5^{-5}$, was employed as an internal standard. Real-time qPCR was performed in 25 μL reactions containing 1 μL of template cDNA or the standard, 2× SYBR Green PCR premix (TaKaRa), and 0.3 μmol/L of each primer. Thermal cycling conditions were as follows: 1 cycle at 94℃ for 15 s, 40 cycles at 94℃ for 10 s, 60℃ for 30 s. Melting curve analysis was performed from 60 to 94℃. The relative molar levels of *HSP*23 and 18S rRNA transcripts were calculated based on crossing point analysis, using standard curves generated from the cDNA standards. *HSP*23 mRNA levels were normalized with those of 18S rRNA in the same samples quantified in the same manner, and the final relative mRNA levels of *HSP*23 were averages of three replicates. The fluorescent real-time PCR assay was carried out in a Bio-Rad iCycler iQ5 (Bio-Rad, USA). Statistical analysis was performed using SPSS 13.0 for Windows. Data were expressed as mean ± *SE* and subjected to a one-way ANOVA, followed by Duncan's test and a Student's test. A $P < 0.05$ was considered to be significant.

## 3 RESULTS

### 3.1 Cloning and sequence analysis of *DaHSP*23

A fragment of 284 bp was amplified using the degenerate primers, and its nucleotide sequence is significantly similar to those of other known *HSP*23s in GenBank (E value, $8e^{-66} - 3e^{-7}$). The 94 a.a. sequence encoded by the cDNA also has high similarity to known HSP23 protein sequences (E value, $5e^{-40} - 5e^{-24}$). Therefore, the cDNA fragment was determined to be the partial sequence of *HSP*23 gene. Based on the cDNA sequence obtained, the gene-specific primers were synthesized for 3′-end and 5′-end RACE. And then, a 500 bp fragment was amplified by 3′ RACE and a 480 bp fragment by 5′ RACE. The full-length cDNA sequence of *DaHSP*23, which has been deposited in GenBank under accession number HQ392521.1 and ADX36150.1, was obtained by alignment analysis of the above three fragments (Fig. 1).

For *DaHSP*23 intron analysis, the genomic DNA sequence for *DaHSP*23 was amplified by specific primers (CDS23F, CDS23R), and then was compared to cDNA sequence for *DaHSP*23. The result showed that these two sequences are identical, indicating that there is no intron in the coding region of *DaHSP*23 gene.

The full-length *DaHSP*23 cDNA, 904 bp in length, encoding a 186 a.a. peptide with a calculated molecular weight of 20.9 kDa and a theoretical isolectric point of 6.42, contains a 5′-terminal untranslated region (UTR) of 132 bp, a 3′-terminal UTR of 211 bp and an open reading frame (ORF) of 561 bp. A polyadenylation signal sequence AATAAA is located at nucleotides 710 – 715 (Fig. 1). The alignment of DaHSP23 with other five homologous dipteran sHSPs (DmHSP20.6, DbHSP20.6, CcHSP18.9, GmHSP23.1 and ScHSP23.0) is shown in Fig. 2. The conserved α-crystallin domain

is predicted to consist of a. a. 65 to 142, which is characteristic of sHSPs. The InpA region for posttranslational modification, protein turnover and chaperones is predicted to consist of a. a. 12 to 158, based on *D. melanogaster* (AAN11962. 1). The putative dimer interface sites are predicted to be at a. a. 71 (C), a. a. 73 (D), a. a. 75 – 76 (QQ), a. a. 113 (R), and a. a. 134 – 135 (GV) (Fig. 2).

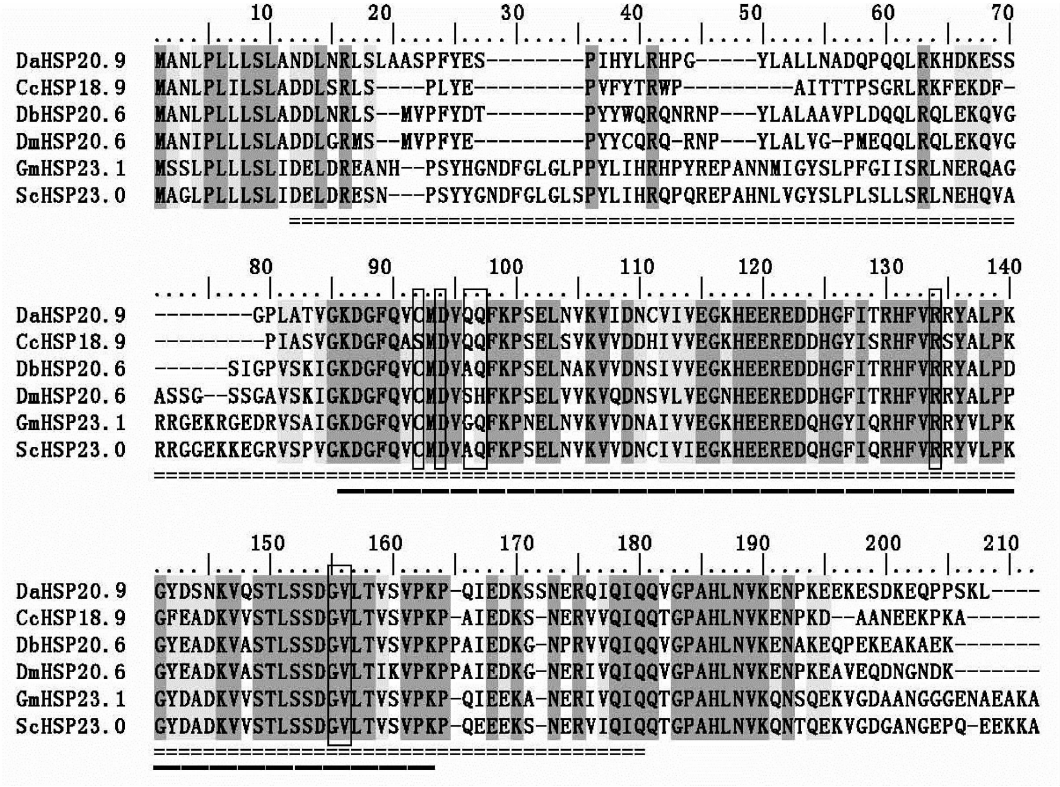


Fig. 2 Alignment of DaHSP23 (DaHSP20.9 in the figure) with other five homologous dipteran sHSPs

The conserved α-crystallin domain is underlined, the InpA region is double underlined, and the putative dimer interface sites are boxed. The amino acids with 100% and over 70% identity are shaded with dark and gray color, respectively. A "-" corresponds to a gap and the residue column is each assigned a number above. The accession number of each sequence is same as in Fig. 3.

### 3.2 Homology and phylogenetic analysis of DaHSP23

The global search with BlastP in GenBank non-redundant database using DaHSP23 as query retrieved 18 homologues with E-value larger than $4e^{-50}$ and 100 with E-value larger than $1e^{-26}$. Of the 100 homologues, 28 belong to Diptera, and 10 representative sequences (Table 2) with full length were finally selected to perform further sequence and phylogenetic analysis with DaHSP23. The DaHSP23 showed the highest identity (68. 2%) with DvSHP20. 7 (*Dr. virilis*) and DbHSP20. 6 (*Dr. buzzatii*), followed by CeHSP18. 9 (65. 1%, GenBank accession no. ACG58884. 1), DmHSP20. 6 (64. 9%, GenBank accession no. NP_523999. 1) and DpHSP20. 4 (64. 4%, GenBank accession no. XP_002024748. 1) (Table 2).

The phylogenetic tree of DaHSP23 and the other 10 dipteran homologous sHSPs was constructed, with the homologous sHSP of *C. elegans* as the outgroup (Fig. 3). Six sHSPs from six species of *Drosophila*, two sHSPs of *Ceratitis capitata*, and two sHSPs from *Glossina morsitans morsitans* and *Sarcophaga crassipalpis* were separately clustered into three monophyletic clades, all with 100% bootstrap support.

### 3.3 Expression of *DaHSP*23 during summer and winter diapause

The relative mRNA levels of *DaHSP*23, normalized to 18S rRNA levels, were very low in both pre-diapause (0. 85 and 1. 46 for SD0. 5 and SD1, respectively) and post-diapause (2. 19 for SD17) stages (Fig. 4). At the onset of SD (SD5), the level increased to 3. 29, and then rapidly peaked (4. 50, SD10). Thereafter, the level gradually decreased from 6. 04 (SD11) to 2. 19 (SD17), with a slope rate of −0. 64/d (Fig. 4). The relative mRNA levels of *DaHSP*23, normalized to 18S rRNA levels, were relatively low in the pre-diapause stages [0. 97(WD3), 1. 06(WD4)]. During the diapause, the level rapidly increased from 2. 98 (WD29) to 8. 01 (WD54), and then gradually decreased to 2. 33 (WD103), and then to 2. 22 (WD114), with a slope

**Table 2　The dipteran sHSPs homologous to DaHSP23 and used for sequence and phylogenetic analysis, and their identity and similarity compared with DaHSP23**

| sHSP name | Species | GenBank accession number | Amino acid sequence identity (%) | Amino acid sequence similarity (%) |
|---|---|---|---|---|
| DmHSP20.6 | *Drosophila melanogaster* | NP_523999.1 | 64.9 | 74.7 |
| DpHSP20.4 | *D. persimilis* | XP_002024748.1 | 64.4 | 74.7 |
| DwHSP20.8 | *D. willistoni* | XP_002062617.1 | 61.5 | 76.6 |
| DvSHP20.7 | *D. virilis* | XP_002048201.1 | 68.2 | 79.7 |
| DbHSP20.6 | *D. buzzatii* | ABX80640.1 | 68.2 | 79.2 |
| DgHSP20.4 | *D. grimshawi* | XP_001985085.1 | 66.0 | 77.3 |
| CeHSP18.9 | *Ceratitis capitata* | ACG58884.1 | 65.1 | 79.6 |
| CcHSP18.9 | *C. capitata* | ACG58883.1 | 62.9 | 77.4 |
| GmHSP23.1 | *Glossina morsitans morsitans* | ADD18976.1 | 53.1 | 66.4 |
| ScHSP23.0 | *Sarcophaga crassipalpis* | AAC63387.1 | 57.0 | 69.1 |
| Outgroup | *Caenorhabditis elegans* | NP_001023957.1 | 30.3 | 45.7 |

sHSP sequence names are suffixed with deduced molecular weights in kDa.

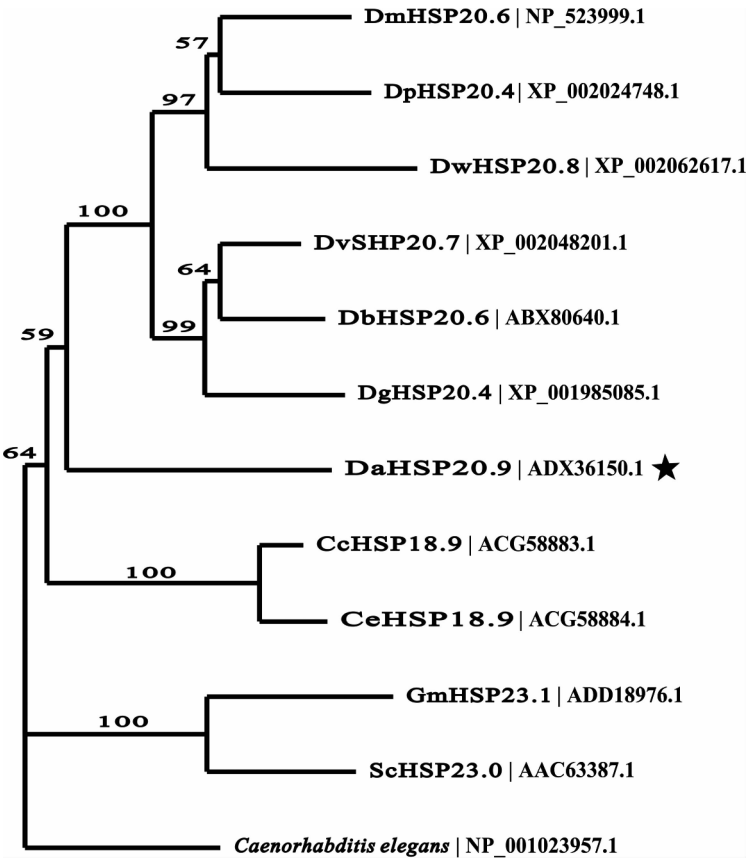


Fig. 3　The neighbor-joining (NJ) tree of eleven dipteran sHSPs

The homologous sHSP of *Caenorhabditis elegans* (GenBank accession number: NP_001023957.1) was used as the outgroup, and percentage bootstrap values are indicated on each node. Dm: *Drosophila melanogaster*; Dp: *D. persimilis*; Dw: *D. willistoni*; Dv: *D. virilis*; Db: *D. buzzatii*; Dg: *D. grimshawi*; Ce: *Ceratitis capitate*; Cc: *C. capitate*; Gm: *Glossina morsitans morsitans*; Sc: *Sarcophaga crassipalpis*.

rate of −0.09/day (Fig. 4). In brief, the relative mRNA levels of *DaHSP*23 in the diapause stages (WD29, WD54, and WD79) were much higher than those in pre-diapause (WD3, WD4) and post-diapause stages (WD103, WD114) ($P < 0.05$) (Fig. 4). These results indicated that the expression of *DaHSP*23 was almost constitutive under unstressed conditions but was rapidly up-regulated during winter diapause. The relative mRNA levels of *DaHSP*23 in SD and WD were similarly high compared to those in ND (between 0.94 at ND1 and 1.46 at ND9) (Fig. 4). The relative mRNA levels of *DaHSP*23 in pre-diapause and post-diapause of SD and WD were similarly low compared to those in ND (Fig. 4).

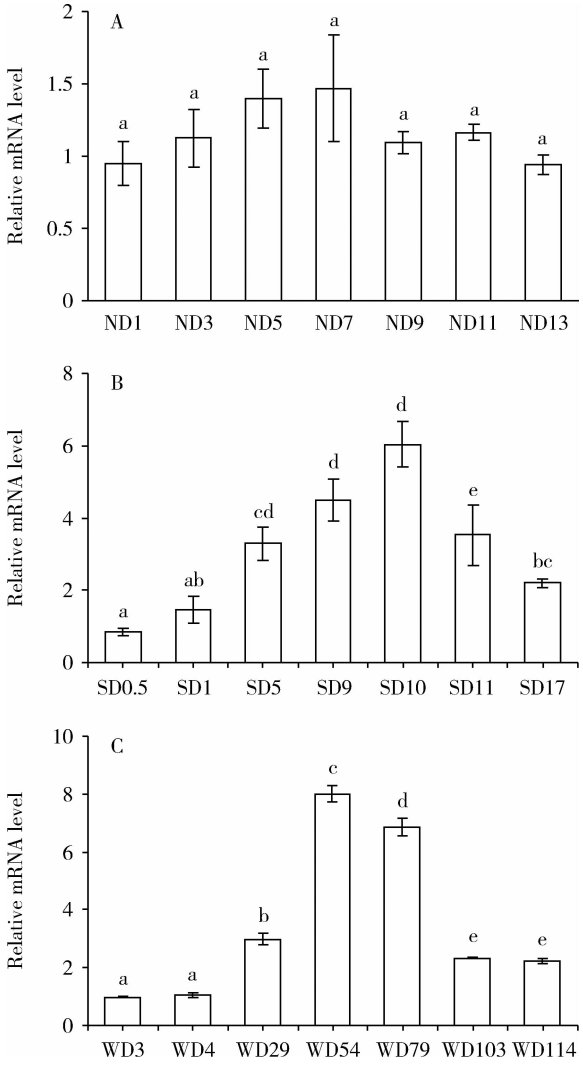


Fig. 4 Real-time qPCR analysis of *DaHSP*23 mRNA levels in non-diapausing (ND) (A), summer diapausing (SD) (B) and winter diapausing (WD) (C) pupae of *Delia antiqua*

Relative mRNA levels were normalized to the internal standard 18S rRNA, with the bars indicating the mean ± *SE* of three replicates and different letters between samples demonstrating the significant transcript difference ($P \leqslant 0.05$). Data were expressed as mean ± *SE* and subjected to a one-way ANOVA, followed by Duncan's test and a Student's test. ND1, SD0.5 and WD3 represent day 1, day 0.5 and day 3 after pupation of ND, SD and WD, respectively.

# 4 DISCUSSION

The amino acid sequence of DaHSP23 contains an alpha-crystallin domain (ACD) and a hallmark, which is a characteristic of the sHSP family (De Jong *et al.*, 1998; McHaourab *et al.*, 2009). The sequence exhibits high conservation with other dipteran HSP23s, and shares the highest similarity with those reported to be related to diapause.

In the HSP70 family, introns are generally found only in constitutively expressed genes but not in the inducible genes (Ming *et al.*, 2010). In sHSP family, most members are intronless (Caspers *et al.*, 1995). It was reported that the lack of introns in inducible HSP genes could help to circumvent a block in RNA splicing and enable preferential expression of the heat shock proteins during periods of stress (Yost and Lindquist, 1986; Huang *et al.*, 1999), thus protecting cells against harmful insults. The genome sequencing of *DaHSP*23 in the present study showed that it is intronless, suggesting that it is inducible and qualified to be involved in diapause development and stress response.

In *D. antiqua*, the expression of *DaHSP*23 was gradually up-regulated with SD and WD development, and declined to pre-diapause levels when SD and WD diapauses were terminated. The WD result was comparable to that found for *HSP*23 in WD pupae of *S. crassipalpis* (Yocum *et al.*, 1998). This is the first study on *HSP*23 expression in response to SD. Either cold- or heat-shock can upregulate the expression of inducible *HSPs* (Yocum *et al.*, 1998), and up-regulated expression is evident for diapausing pupae that are not subject to temperature stress. This implies that expression of the sHSP protein is a component of the diapause syndrome in this species. Earlier studies suggest that small heat shock proteins are involved in the regulation of cell cycle arrest in diapause. Mounting evidence indicates that small heat shock proteins serve a pivotal role in cell growth and differentiation. If the small heat shock proteins are involved in cell cycle arrest associated with diapause, they are functioning in concert with other cell cycle regulator (Yocum *et al.*, 1998).

In contrast, the expression of *HSP*23 is not up-regulated as a function of diapause in *D. triauraria* and *Lucilia sericata* (Goto and Kimura, 2004; Tachibana *et al.*, 2005). Thus, the up-regulation of the expression of *HSP*23 might not be a common feature of diapause insects. Variations among species are also found in the expression of other HSP genes such as *HSP*70 and *HSP*90. The expression of these genes was not regulated in *D. triauraria* and *L. sericata* during WD (Goto *et al.*, 1998; Goto and Kimura, 2004; Tachibana *et al.*, 2005), whereas that of *HSP*70 was highly up-regulated in *D. antiqua* during WD (Chen *et al.*, 2006) and that of *HSP*90 was down-regulated in *S. crassipalpis* (Yocum *et al.*, 1998; Rinehart and Denlinger, 2000). These results suggest that gene expression in response to diapauses might be species-specific.

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# 葱蝇热激蛋白 DaHSP23 基因的克隆及在冬滞育和夏滞育蛹中的表达分析

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**摘要:** 【目的】低分子量(12 ~ 43 kDa)热激蛋白(sHSPs)具有抗逆应答的功能,滞育是昆虫抵抗不良环境的特殊发育形式,但 sHSPs 在昆虫滞育发育过程中的作用仍不清楚。本研究克隆和特征化葱蝇 *Delia antiqua sHSP* 基因,并研究它在夏滞育和冬滞育发育过程中的表达模式,为阐明 sHSPs 在滞育发育上的功能奠定基础。【方法】通过 RACE-PCR 方法克隆了葱蝇 HSP23 基因,通过相似性比较分析了其特征、结构域及与双翅目代表性同源基因的系统发育关系;采用实时荧光定量 PCR 研究了该基因在葱蝇冬滞育蛹和夏滞育蛹发育过程中的表达情况,通过表达的差异比较揭示了该基因与滞育发育的关系。【结果】克隆出了葱蝇 HSP23 基因,命名为 *DaHSP*23 (GenBank 登录号: HQ392521.1),其 cDNA 全长序列为 904 bp,编码 186 个氨基酸,推测蛋白分子量为 20.9 kDa,等电点为 6.42。该基因的编码蛋白与其他双翅目昆虫的 sHSPs 有超过 66% 的氨基酸序列一致性,与已报道的其他双翅目昆虫的滞育相关 HSP23 基因同源。基因组测序显示该基因无内含子。*DaHSP*23 基因在葱蝇非滞育蛹的发育过程中一直保持在较低的水平,各发育阶段间的表达量不存在显著差异。但在冬滞育和夏滞育蛹中,该基因从滞育起始期开始逐渐显著升高表达,到滞育维持期的中后期达到峰值,在滞育终止期逐渐降到较低的水平。【结论】*DaHSP*23 基因在葱蝇冬滞育和夏滞育发育过程中明显上调表达,但存在差异,它在滞育期的调控可能是种专化的。*DaHSP*23 可能在葱蝇两种类型的滞育上起重要作用。

**关键词:** 葱蝇; 滞育; 热激蛋白; 基因克隆; 表达模式; 实时荧光定量 PCR

**中图分类号:** Q966 **文献标识码:** A **文章编号:** 0454-6296(2016)04-402-09

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